SYNOPSIS OF THE PH.D. THESIS

Tiitled:

"STUDIES ON MOUTH PARTS AND GUT PHYSIOLOGY OF WORKER HONEY BEE *Apis cerana indica Fabricius*, 1798".

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INTRODUCTION

Honey bees play a vital role in both honey production and maintaining food security and biodiversity by pollinating plants and supporting various ecosystems. However, urbanization and pesticide use have led to a decline in bee populations, posing a significant threat to ecosystems worldwide. Without bees, not only would honey production be impacted, but biodiversity and global food security would also suffer, highlighting the critical importance of these pollinators.Throughout history, people have recognized the therapeutic properties of bee-related products. There is growing evidence suggesting that these items, such as honey, possess anti-inflammatory, antimicrobial, antioxidant, and potentially even anti-cancer effects. Honey, in particular, has been used in traditional medicine to treat a wide range of ailments, including eye diseases, asthma, throat infections, tuberculosis, fatigue, and skin conditions like eczema and ulcers. Beeswax, historically used for waterproofing and fuel, now offers health benefits and is commonly found in skincare products and pharmaceutical ointments. Other bee products, such as propolis, bee pollen, royal jelly, and bee venom, also provide various health advantages. In a recent study in 2020, scientists discovered evidence suggesting that melittin, a component of honeybee venom, may have the potential to eliminate cancer cells (Millar, 2021).

The honey bee's mouthparts, situated in the lower portion of the head, facilitate two distinct actions: chewing and lapping, tailored for collecting nectar and pollen. These mouth components include the labrum, epipharynx, mandibles, maxillae, and labium. The labrum, positioned below the clypeus, precedes the fleshy epipharynx, serving as a taste organ. Mandibles, smooth and flanking the labrum, shape wax and construct honeycomb. The labium comprises elongated labial palps on each side, along with the submentum, mentum, paraglossa, and glossa, or tongue. The glossa, an extendable tool, facilitates honey collection, featuring a small labellum at its tip. Maxillae, equipped with minuscule maxillary palps, align with the mentum on either side. During nectar gathering, the glossa maneuvers within a tube formed by the maxillae and labial palps, coating its surface with nectar as it ascends. Research suggests that this coating phenomenon may be attributed to wetting and capillarity, akin to the uptake of liquid by a brush-tipped proboscis on wet surfaces (Lee and Lee, 2014).

Honey bees rely on a diverse range of flowering plants for pollen and nectar, essential for honey production. Pollen, rich in nutrients including sugar, carbohydrates, protein, enzymes,

vitamins, and minerals, serves as a vital component of their diet. Honey, produced from collected nectar, serves as the primary food for honey bee larvae, with potential queen larvae exclusively fed royal jelly, a substance rich in nutrients and reproductive boosters. The consumption of royal jelly accelerates the development of queen bees, extending their lifespan significantly.Pollen, the primary source of protein for bees, also contains lipids, vitamins, and minerals crucial for the development of young bees and brood production. However, not all pollen is nutritionally equal, and variations in its quality impact bee health and colony productivity. Insufficient or low-quality pollen can lead to decreased brood rearing and shortened worker lifespans, ultimately affecting colony productivity and survival. Foragers collect pollen using their front and middle legs, transferring it to specialized structures on their hind legs known as pollen baskets. Once collected, pollen is deposited into cells within the hive and transformed into a paste-like consistency through the actions of other worker bees. Fermentation by bee secretions further enhances its nutritional value. Nectar, the primary source of carbohydrates, is collected by honey bees using their proboscis and stored in their honey crop. The crop acts as a reservoir for nectar, connecting to the midgut for digestion. It also filters pollen from nectar and prevents contamination of honey or nectar. Additionally, the honey crop synthesizes ethyl oleate, a pheromone signaling young bees not to transition into foragers. (Calderone and Page, 1992).

Bees primarily encounter pesticides through direct contact and ingestion of pollen or nectar, their main nutritional sources comprised of nectar, honey, pollen, and bee bread—a mixture of stored pollen and nectar or honey. Herbicides and fungicides are typically applied to the soil before crop planting, while insecticides are sprayed over crop canopies. These treatments release tiny droplets and dust particles, which can settle on bees flying through or near treated fields, potentially killing them due to the concentrated dosage (Marinelli *et al*., 2004; Smith *et al*., 1994). Systemic insecticides, applied as seed coatings, produce insecticide-laden dust particles when distributed into the soil using pneumatic drilling planters, posing risks to bees present during planting (Georgiadis *et al.*, 2012). Additionally, systemic insecticides absorbed by crop plants during growth accumulate in all plant parts, including flowers, pollen, and nectar, further exposing bees (Bonmatin *et al*., 2005). Guttation drops, high in systemic insecticides, produced by certain plants like maize and strawberries in the morning, can also harm bees (Tapparo *et al*., 2011). Bees forage on pollen and nectar from various sources, including agricultural plants and surrounding weeds, accumulating pesticide residues that persist in their colonies' beebread and

honey (Kubik *et al*., 1999; Orantes-Bermejo *et al*., 2010). Water sources in agricultural areas can also contain pesticide residues from soil runoff and spray drift, further exposing bees as they consume contaminated water (Belden *et al*., 2007; McCune *et al*., 2021). This multi-pronged exposure poses significant risks to bee populations and ecosystems (Bayo and Goka, 2014).

Natural honey, primarily comprised of sugars (glucose and fructose), water, protein, ash, and trace elements of vitamins, enzymes, amino acids, and phenolic antioxidants, exhibits diverse chemical and physical qualities influenced by nectar and pollen sources, color, flavor, and moisture content (White, 1975; White & Maher, 1980; Barth, 1989). Sugars account for about 82%, including fructose (38.2%), glucose (31%), and various disaccharides and oligosaccharides. Honey contains enzymes like invertase, amylase, glucose oxidase, catalase, and acid phosphorylase, along with 18 free amino acids and trace B vitamins, minerals, and ascorbic acid. Flavonoids, including pinocembrin unique to honey, serve as primary antioxidants, enhancing with honey's color. Honey's sensory and physiochemical properties vary based on environmental factors and plant origin, impacting phenolic content and antioxidant capacity (Gheldof & Engeseth, 2002; Turkmen *et al*., 2005; Bertoncelj *et al*., 2007). Acacia and lime honey show lower phenolic content than other varieties, affecting antioxidant benefits (Socha *et al*., 2009).

OBJECTIVES

- **To study the of ultrastructure mouthparts of honey bees (*Apis cerana indica Fabricius*).**
- **To study foraging preferences of honey bees (*Apis cerana indica Fabricius*) by palynology.**
- **To study effects of pesticides and fungicides in honey bees (*Apis cerana indica Fabricius*) on gastrointestinal motility and gut pH.**
- **To study the constituents of honey with reference to protein content, colour, phenolic, flavanoid content and antioxidant activity of honey and compare urban and rural samples.**

The results of the work done were compiled and organized in the following chapters;

Chapter I : General introduction and Review of literature

Chapter II : Studies on mouth parts of Honey Bee (*Apis cerana indica*)

Chapter III: Foraging preferences of *Apis cerana indica* in Western Ghats at some sites of Dakshina Kannada – A palynological study

Chapter IV: Gastrointestinal motility and pH: Effect of pesticides and fungicides.

Chapter V : Protein content, colour, flavanoid, phenolic and antioxidant activity of honey from Dakshina Kannada.

STUDIES ON MOUTH PARTS OF HONEY BEE (*Apis cerana indica*)

MATERIAL AND METHODS

The study involved the microscopic examination of Asian worker honey bees, Apis cerana indica, collected from apiaries located in Puttur, Karnataka, India (12.7687° N, 75.2071° E, 87 m elevation), situated at the base of the Western Ghats. A total of fifty honey bees were gathered from various colonies, and from these, seven bees were randomly chosen for detailed scanning electron microscopy (SEM) analysis.The procedure commenced by carefully dissecting the bees using fine forceps under a dissecting microscope to extract their mouthparts. Subsequently, the excised mouthparts were immersed in a solution of 4% glutaraldehyde (dissolved in 1 M phosphate buffer) for a duration of four hours. To ensure cleanliness, any debris on the bees was removed through a brief period of ultrasonic cleaning (using the Sidilu Ultrasonic device from Bangalore) for 5 seconds. The mouthparts underwent a thorough cleansing process, first with distilled water and then through a series of steps to dehydrate them. Following this, whole mounts were meticulously prepared and secured in place using DPX.

Scanning Electron Microscopy

The collected samples were preserved using either absolute alcohol or Karnovsky's fixative (Karnovsky, 1964). These specimens underwent dehydration by passing through a series of alcohol concentrations. Following a final rinse in acetone, the samples were coated with a 5 nm thick layer of gold using a Class I gold sputtering system. Subsequently, they were examined

utilizing a field emission scanning electron microscope (FESEM) from Carl Zeiss Ltd., Germany.

The resulting images were processed using Adobe Photoshop software. To categorize the sensilla, descriptions from Callahan (1975) and those specific to *Apis mellifera* by Whitehead & Larsen (1976b) were used as references for naming and identification purposes.

Silver Nitrate Staining

Porous chemosensilla were specifically treated with silver nitrate using a technique outlined by (Babu *et al*., 2011) and examined using an Olympus BX51 microscope. To begin, the bees were anesthetized and then washed with 1% acetone. Following this, they were immersed in a 1% silver nitrate (AgNO3) solution for a duration of five minutes. Afterward, the bees underwent a triple two-minute wash to eliminate any residual AgNO3.The next steps involved treating the specimens by soaking them in a photo/film developer for a period of five to seven minutes. Subsequently, the specimens were swiftly rinsed in 3% acetic acid for approximately one minute. The specimens then went through a dehydration process involving successive immersions in 70%, 80%, 90%, and finally 100% alcohol for durations of 10 minutes each. Once dehydrated, the samples were washed in methyl salicylate. Finally, the antenna or maxillary palp was mounted on a slide using DPX, a mounting medium, to facilitate examination under the microscope.

Nomenclature of Sensilla

The nomenclature for sensilla is based on the descriptions provided by Callahan (1975) and Whitehead & Larsen (1976b) specifically pertaining to Apis mellifera. However, the naming system for sensilla in A. florea, as indicated by (Kumar & Kumar, 2016), appears to be unclear. Their classification might lack specificity or could be subject to differing interpretations, leading to uncertainty in identifying and labeling sensilla in A. florea.

RESULTS

The sensory structures present on different segments of the mouthparts—such as the glossa, labellum, labial palp, galea, and maxillary palp—are described here in detail. The glossa and labellum together measure approximately 1.8 ± 0.1 mm in length and 120 ± 8 μm in width. The

oval-shaped labellum measures 120 ± 7µm by 80 ± 6µm. The glossa contains hairs that aid in collecting nectar through surface tension and capillarity. On the labellum's dorsal side, there are forked hairs, while the ventral surface lacks hair . The grooved labellum assists in nectar suction and capillary action facilitates the drawing-in of nectar. The glossa is enclosed by the labial palp and galea. Various types of sensilla observed include Sensilla chaetica (Type A and Type B, based on length), Sensilla basiconica, and Sensilla coeloconica.

At the base of the labellum, there are six sensilla chaetica on each side, slightly curved and measuring 40 ± 4 µm in length. They stain positively with silver staining, indicating their likely function as chemosensory and possibly gustatory structures . Along the distal two-thirds of the glossa, shorter sensilla measuring 30 µm in length were observed, also silver-positive. The glossa is covered with numerous thin microtrichia (120 ± 9 µm long and 2 ± 0.12 µm wide) placed approximately 20 ± 1.1 µm apart from each other, aiding in nectar collection.The labial palp consists of four segments, with segments 3 and 4 protruding from the galea. Segments 1 and 2's length enables their protrusion, and segments 2, 3, and 4 possess chemosensilla. These sensilla types—Sensilla chaetica A, Sensilla chaetica B, and Sensilla basiconica—are notably stained by silver, primarily at their tips. The galea en-sheaths the labial palp and glossa and bears sensilla coeloconica at the proximal region, along with Sensilla chaetica A (12 ± 1µm) and Sensilla chaetica B (20 ± 1.8 µm) . Additionally, the ventral surface of the galea exhibits four sensilla basiconica, a type previously described in A. mellifera or A. florea. The Sensilla chaetica have been identified as chemosensory due to their permeability to silver nitrate.

Moving to the maxillary palp, the second segment bears nine non-porous mechanosensory hairs measuring between 12 µm to 24 µm. The bulged surface at the galea's base near the maxillary palp bears a group of 34 ± 2 silver-permeable sensilla basiconica measuring 2 ± 0.2 µm . Lastly, the mandibles possess numerous microtrichia measuring 10 µm, likely serving as mechanosensory structures due to their lack of staining by silver nitrate.

FORAGING PREFERENCES OF *APIS CERANA INDICA* IN WESTERN GHATS AT SOME SITES OF DAKSHINA KANNADA – A PALYNOLOGICAL STUDY

MATERIALS AND METHODS

Between February 2016 and April 2016, 14 honey samples were collected from various locations within the Dakshina Kannada District, covering an area from 12.520 N & 74.530 E to 12.870 N & 74.880 E in Karnataka, India, encompassing an area of 4,559 km² (Dakshina Kannada District Profile, 2015). These samples were obtained from beekeepers across different areas to discern the pollen preferences of honeybees in the region. The geographical coordinates of the honey samples collected ranged in elevation from 55 to 135 meters. Samples were gathered from multiple domesticated *Apis cerana indica* colonies and were in their raw, unprocessed state, extracted using honey extracting machines from honeycombs.

For pollen analysis, each honey sample (5g) was dissolved in 5ml of distilled water, centrifuged at 2,500rpm for 10 minutes, and then treated with 5ml of glacial acetic acid. Following centrifugation, the sediment containing pollen was subjected to acetolysis according to Erdtman's method (1952). The resulting material was mixed with glycerin jelly, placed on a glass slide, slightly heated to melt the jelly, and covered with a cover slip secured by colorless nail polish (Song *et al*., 2012; Ponnuchemy *et al*., 2014).

Qualitative and quantitative analysis of the pollen in the honey samples involved preparing three pollen slides per sample. Pollen types were identified by comparing them with reference slides from plants within the study area and using existing palynological literature. For quantification, 300 pollen grains from each sample were counted and classified into four frequency classes: predominant (>45%), secondary (16–45%), important minor (3–15%), and minor (<3%) pollen types (Louveaux *et al*., 1978; Yansong *et al*., 2012). Absolute pollen counts per 1g of honey were also determined. Light microscopy was used to observe pollen grains, while scanning electron microscopy was employed for predominant pollen types.

RESULTS

Among the 14 honey samples obtained, 12 distinct pollen morpho-types were identified. Eleven samples predominantly contained pollen from a single flower source, while three were multi-floral. In samples from Irde (DK-Ir-01), Mithanadka (DK-Mi-05), and Mitoor (DK-Mt-

14), Ixora coccinea constituted the primary pollen, ranging from 46% to 60%. Bettampady (DK-Be-03) and Kadaba (DK-Ka-09) samples were predominantly Cocos nucifera, comprising 50% to 60% of the pollen. Samples from Mudipu (DK-Mu-06), Belvai (DK-Bl-08), and Kundadka (DK-Ku-11) exhibited predominant Psidium guajava pollen, ranging from 60% to 68%. Areca catechu was the primary pollen in the Deppuni sample (DK-Dp-10), accounting for 48%, while Mimosa pudica dominated in Derlakate (DK-De-07) and Uppinangady (DK-Up-13) samples, ranging from 50% to 68%. Several pollen types, including *M. pudica, P. guajava, A. catechu, C. nucifera, and I. coccinea*, emerged as significant forage sources for honey bees in Dakshina Kannada.

Certain pollens like *A. catechu* in DK-Ka-09 and DK-Be-03, *C. nucifera* in multiple samples, *I. coccinea* in DK-Pa-04, *M. pudica* in DK-Ke-02 and DK-Ku-11, *Moringa olifera* in DK-Ir-01 and DK-Pa-04, *P. guajava* in DK-Pu-12 and DK-Ke-02, and *Syzigium* in DK-De-07 were secondary sources. Others were observed as minor pollen in specific samples.. Absolute pollen counts per gram of honey revealed DK-De-07 with the minimum count of 1,740 grains and DK-Mi-05 with the maximum count of 27,300 grains. The percentage distribution of various plant species' pollen in each honey sample was calculated. *Cocos nucifera*, present in all samples, accounted for the maximum at 30.142%. *Acacia sp.*, *Mangifera indica*, and *Eucalyptus sp.* registered the lowest pollen percentages.

Identified pollen sources encompassed 12 plant species across seven families. *Arecaceae* and *Myrtaceae* each contributed three species, while *Fabaceae* and several other families contributed one species each.. *Cocos nucifera* emerged as a vital pollen source, present in all 14 samples, underscoring its significance in Dakshina Kannada.

The mean absolute pollen count of honey samples collected from hills was 3240±991.72, from plantation was 12644±10019.92, from plains was 2056.67±438.55. A **one way ANOVA** showed that there was a significant difference in the mean abundance of absolute pollen count{$F(2,39) = 12.79$, $p < 0.01$}. The mean abundance of absolute pollen count of honey has shown significant difference which is <0.01.A **tukey HSD post hoc** test revealed that the mean absolute pollen count abundance in honey samples of plantation was significantly higher than hills as well as plain regions ($p<0.01$). However, there was no difference in the abundance between hills and plains ($p=0.881$).

GASTROINTESTINAL MOTILITY AND PH: EFFECT OF PESTICIDES AND FUNGICIDES.

MATERIAL AND METHODS

Adult worker honey bees (*Apis cerana indica*) were collected from two distinct colonies in Mangalore. They were transported to the laboratory and given a feed comprising 1ml of honey mixed with 100 µl of Bromophenol blue pH indicator. After an hour of this feeding, the honey bees were anesthetized using ether and subsequently dissected under a stereo microscope. The examination of the honey bee's digestive system allowed for the observation of the color in the intestinal tract, aiding in the determination of pH levels. This experiment was then replicated by supplementing the honey feed with 100 µl of three different sub-lethal doses of pesticides (Cypermethrin, Cyhalothrin) and fungicides (Copper sulfate, Bordeaux mixture, Mancozeb). Each pesticide and fungicide was separately added to the feed and administered to individual honey bees, noting any variations in the pH indicator's color within the intestine.

To investigate gastrointestinal motility, honey bees were deprived of food for 1 to 2 hours and subsequently fed a 1ml honey solution containing 100 µl of Bromophenol blue. Following the feeding, they were isolated in separate containers for a specific duration (20 minutes). After this period, their alimentary canals were extracted as per the method outlined by Whitcomb & Wilson (1929), observed, and photographed under a stereo microscope. The stained food's distance traveled through the alimentary canal was measured. This experiment was repeated by introducing 100 µl sub-lethal doses of the aforementioned pesticides and fungicides into the honey feed, separately administered to the honey bees. The alimentary canal was then observed and photographed under the stereo microscope, noting any variations in gastrointestinal transit time.

RESULTS

In order to investigate the impact of pesticides like 0.1% Cypermethrin and fungicides such as Copper sulfate and 0.6% Mancozeb on the intestinal pH of worker honey bees, a pH-sensitive dye, bromophenol blue, was utilized. Worker honey bees were gathered from two distinct colonies and transported to the laboratory. They were then fed a mixture of honey and Bromophenol blue. This particular dye serves as an acid-base indicator, exhibiting its

effectiveness within a pH range of 3.0 to 4.6. Bromophenol blue transitions from a yellow hue at pH 3.0 to a blue shade at pH 4.6 (Erban and Hubert, 2010).

Empty digestive tract was observed without any food material or dye plate . Control was prepared by feeding food along with Bromophenol blue indicator which showed midgut with pH >4.6 plate , The honey bee was fed with food added with bromophenol blue and1% Bordeaux mixture showed pH <4 plate1(c), when food with bromophenol blue was added with 0.5% Bordeaux mixture, the midgut showed pH >4 plate1 (d), food with bromophenol blue added with 0.25% Bordeaux mixture showed pH >4.6 plate 1(e).

Digestive tract with food added with bromophenol blue and 1% CuSo4 showed midgut with pH <4, The honey bee was fed with food added with bromophenol blue and 0.5% CuSo4 showed pH >4, when food with bromophenol blue was added with 0.25% CuSo4 ,the midgut showed pH >4.6,food with bromophenol blue was added with 0.1% Cyhalothrine showed pH <4, By adding bromophenol blue to food and 0.05% Cyhalothrine showed pH >4, food with bromophenol blue added with 0.025% Cyhalothrine showed pH >4.6.

Digestive tract of honey bee fed with food added with bromophenol blue and 0.1%Cypermethrine showed pH <4, When honey bee was fed with food added with bromophenol blue and 0.05% Cypermethrine, midgut showed pH >4, Food mixed with bromophenol blue and 0.025%Cypermethrine showed pH >4.6,By adding food with bromophenol blue and 0.1% Mancozeb showed pH <4, When food with bromophenol blue was added with0.5% Mancozeb midgut showed pH >4, food with bromophenol blue and 0.25% Mancozeb showed pH >4.6.

Rate of gastrointestinal motility

It also has a pro-kinetic effect increasing the motility of the gut as measured by time taken for movement of food. Gastro intestinal motility was measured for a time interval of 20 minutes. The distance traveled by food inside the digestive tract, was measured in terms of µm. At normal condition the honey mixed with bromophenol blue dye only travelled 4300 µm in a time of 20 minutes. When 1% Bordeaux was added it traveled 5300 µm. In the presence of pesticide 0.1% cypermethrine it moved 4666 µm. When 1 % mancozeb was added the distance traveled was 4630 µm. In the presence of 1% copper sulphate it was 5333 µm. When 0.1% Cyhalothrin was added it traveled 4666 µm.

The mean distance travelled by honey samples mixed with 0.25% Bordeaux was 4678±101.53, 0.50% Bordeaux was 5000±166, 1% Bordeaux was 5300±78. A **one way ANOVA** showed that there was a significant difference in the mean distance travelled by honey samples mixed with bordeaux{$F(2,6) = 19.81$, $p = 0.02$}. The mean abundance of absolute pollen count of honey has shown significant difference which is =0.02.

A **tukey HSD post hoc** test revealed that the mean distance travelled by honey sample mixed with 0.50% bordaeux was significantly higher than 0.25%bordeaux (p=0.04), mean distance travelled by honey sample mixed with 1% bordaeux was significantly higher than 0.25%bordeaux (p<0.01) .However, there was no significant difference in the distance travelled between 1%Bordeaux and 0.50% Bordeaux (p=0.05).

The mean distance travelled by honey samples mixed with 0.25% Copper sulphate was 4333±153, 0.50% Copper sulphate was 4666±57,1% Copper sulphate was 5333±95. A **one way ANOVA** showed that there was a significant difference in the mean distance travelled by honey samples mixed with Copper sulphate {$F(2,6) = 65.40$, $p < 0.01$}. The mean distance travelled by honey samples mixed with three concentrations of Copper sulphate has shown significant difference which is <0.01.

A **tukey HSD post hoc** test revealed that the mean distance travelled by honey sample mixed with 0.50% Copper sulphate was significantly higher than 0.25%bordeaux (p=0.02) and 1% Copper sulphate (p<0.01), mean distance travelled by honey sample mixed with 1% Copper sulphate was significantly higher than 0.25%Copper sulphate (p<0.01) and 0.50% Copper sulphate(p<0.01).

The mean distance travelled by honey samples mixed with 0.025% Cyhalothrin was 4330±111, 0.05% Cyhalothrin was 4400±78,0.1% Cyhalothrin was 4666±86. A **one way ANOVA** showed that there was a significant difference in the mean distance travelled by honey samples mixed with Cyhalothrin {$F(2,6) = 10.96$, $p = 0.01$}. The mean distance travelled by honey samples mixed with three concentrations of Cyhalothrin has shown significant difference which is = 0.01.

A **tukey HSD post hoc** test revealed that the mean distance travelled by honey sample mixed with 0.1% Cyhalothrin was significantly higher than 0.025% Cyhalothrin (p=0.01) and 0.05%

Cyhalothrin(p=0.02). However, there was no significant difference in the distance travelled between 0.05% and 0.025% Cyhalothrin (p=0.64).

The mean distance travelled by honey samples mixed with 0.025% Cypermethrin was 4310±88, 0.05% Cypermethrin was 4400±66,0.1% Cypermethrin was 4666±119. A **one way ANOVA** showed that there was a significant difference in the mean distance travelled by honey samples mixed with Cypermethrin {F(2,6) = 11.74, p <0.01}. The mean distance travelled by honey samples mixed with three concentrations of Cypermethrin has shown significant difference which is <0.01.

A **tukey HSD post hoc** test revealed that the mean distance travelled by honey sample mixed with 0.1% Cypermethrin was significantly higher than 0.025% Cypermethrin (p<0.01) and 0.05% Cypermethrin (p=0.03). However, there was no significant difference in the distance travelled between 0.05% and 0.025% Cypermethrin (p=0.50).

The mean distance travelled by honey samples mixed with 0.25% Mancozeb was 4300±123, 0.50% Mancozeb was 4550±47,1% Mancozeb was 4630±122. A **one way ANOVA** showed that there was a significant difference in the mean distance travelled by honey samples mixed with Mancozeb {F(2,6) = 8.27, p =0.1}. The mean distance travelled by honey samples mixed with three concentrations of Mancozeb has shown significant difference which is =0.1.

A **tukey HSD post hoc** test revealed that the mean distance travelled by honey sample mixed with 1% Mancozeb was significantly higher than 0.25% Mancozeb (p=0.01). However, there was no significant difference in the distance travelled between 0.50% Mancozeb and 0.25% Mancozeb (p=0.05).

PROTEIN CONTENT, COLOUR, FLAVANOID, PHENOLIC AND ANTIOXIDANT ACTIVITY OF HONEY FROM DAKSHINA KANNADA.

MATERIAL AND METHODS

Fourteen honey samples sourced from *Apis cerana* were directly obtained from beekeepers in 2016 across various regions in Dakshina Kannada, Karnataka, India.

Color intensity :

The honey samples underwent dilution to a 50% concentration using distilled water, followed by homogenization and centrifugation at 3000 rpm for 10 minutes. Absorbance measurements were conducted at 635 nm using a spectrophotometer to determine color intensity. The Pfund scale, referenced from Ferreira *et al*. (2009) and Lacerda *et al*. (2010), was employed for assessing the color intensity of the samples.

Total phenolic content

The total phenolic content determination utilized the Folin-Ciocalteu method, expressing results in mg gallic acid/kg honey. Initially, honey solutions were prepared at a concentration of 0.1g/ml. An aliquot of 0.5 ml from this solution was combined with 0.3 ml of Folin-Ciocalteu reagent and 2 ml of 15% sodium carbonate solution. The resulting mixture was adjusted to 5 ml using distilled water and left to incubate at room temperature for 2 hours. Subsequently, the spectrophotometer measured the absorbance of the reaction mixture at 798 nm against a distilled water blank. To establish a standard curve, gallic acid was employed within a concentration range of 7.0x104- to 7.8x 10-3 mg/ml (Socha *et al*., 2007; Ferreira *et al*., 2009).

Flavanoid content

The determination of honey samples' flavonoid content utilized aluminum chloride, expressing the results as mg quercetin/kg honey. The honey solutions were initially prepared at a concentration of 0.2 g/mL. Two milliliters (2 mL) of the stock solution were combined with 3 mL of a 5% aluminum chloride solution. After a 30-minute incubation period, the spectrophotometer measured the absorbance of the reaction mixture at 437 nm against a

methanol blank. To establish a standard curve, quercetin was used within a concentration range of 3.0 x 10–4 to 4.0 x 10–3 mg/mL. (Chang *et al*., 2002).

Antioxidant activity : Total antioxidant capacity.

The Total Antioxidant Capacity (TAC) was determined using the phosphomolybdenum method developed by Prieto Manuel and Miguel (1999). This quantitative assay relies on the reduction of molybdenum (Mo VI) to molybdenum (Mo V) by antioxidants, forming a green complex that reacts with phosphate under acidic conditions, measurable spectrophotometrically at 695nm. In the procedure, 500 µL of honey sample was mixed with 500 µL of 5% Trichloroacetic acid (TCA) to precipitate proteins, followed by centrifugation. After 5 minutes, 100 µL of the clear supernatant was combined with 1 ml of TAC reagent and incubated in a 90˚C water bath for 90 minutes. A blank was prepared using water instead of the sample. Post-incubation, the greenish to bluish color formation was measured at 695nm against the blank. The concentration of total antioxidants in the sample was determined by correlating the absorbance of the test to a standard graph, expressing the concentration as µg/mL.

Total protein estimation by Lowry's method

Prepare different dilutions of BSA solutions by combining the BSA stock solution (1mg/ml) with water in varying proportions as specified in the table. Ensure each test tube contains a final volume of 5ml. The BSA concentrations range from 0.05 to 1 mg/ml. Transfer 0.2 ml of the protein solution from different dilutions into separate test tubes. Add 2 ml of alkaline copper sulphate reagent to each tube and thoroughly mix the solutions. Incubate these solutions at room temperature for 10 minutes. Subsequently, add 0.2 ml of Folin-Ciocalteau reagent to each tube and incubate for an additional 30 minutes. Calibrate the colorimeter using a blank and then measure the optical density (absorbance) at 660nm for each sample. Construct a standard calibration curve by plotting absorbance against protein concentration using the known BSA concentrations. Finally, determine the concentration of the unknown sample by comparing its absorbance value to the standard curve generated earlier. This comparison will help ascertain the protein concentration of the unknown sample (Lowry *et al*., 1951).

RESULTS

The protein content in the honey samples are in the range of 0.754 to 1.240 mg/g. The sample DK-Bl-01 had the highest amount of protein and the sample DK-Ka-05 had the least amount. Other than sample DK-Bl-01 ,samples DK-Mi-03, DK-Pa-04,DK-Mu-07,DK-Be-11,DK-Up-12 and DK-Ke-14 had high protein content (more than 1 mg /g) (Table 1). The protein that pollen provides is very important for the brood production and development of young bees. Pollen is also the main source of protein to honey bees (Ellis *et al.,* 2010).In honey samples biochemical factors which showed significant positive correlations were, protein with Antioxidants; antioxidants with phenolic content and flavanoid content; phenol with flavanoid . However no significant correlation was observed between flavanoid content and other biochemical factors.

There was a significant difference in protein content of honey samples of urban and rural areas(t=2.86,p=0.04). Protein abundance was comparatively higher in honey samples of rural areas (1.04±0.25) than urban areas (0.82±0.11). There was a significant difference in Antioxidant content of honey samples of urban and rural areas (t=2.98,p=0.29). Antioxidant abundance was comparatively higher in honey samples of rural areas (223.10±47.78) than urban areas (177.25±36.46). There was a significant difference in phenol content of honey samples of urban and rural areas (t=-0.670,p=0.27). Phenol abundance was comparatively higher in honey samples of urban areas (1265.75±423.47) than rural areas (1174.53±388.72). There was a significant difference in Flavanoid content of honey samples of urban and rural areas(t=0.532,p=0.62). Flavanoid abundance was comparatively higher in honey samples of rural areas (493.96±152.58) than urban areas (466.41±148.83).

There was a significant difference in protein content of honey samples of 2016-17 and 2021-22 (t=1.51,p=0.61). Protein abundance was comparatively higher in honey samples of 2016-17 (0.98±0.24) than 2021-22 (0.90±0.22). There was a significant difference in Antioxidant content of honey samples of 2016-17 and 2021-22(t=0.53,p=0.98). Antioxidant abundance was comparatively higher in honey samples of 2016-17 (210±49.10) than 2021-22 (204±49.79). There was a significant difference in phenol content of honey samples of 2016-17 and 2021-22 (t=-0.08,p=0.99). Phenol abundance was comparatively higher in honey samples of 2016-17(1200±395.89) than 2021-22 (1193±395). There was a significant difference in Flavanoid

content of honey samples of 2016-17 and 2021-22 (t=0.22,p=0.95).Flavanoid abundance was comparatively higher in honey samples of 2016-17 (486±150) than 2021-22 (478±153).

The protein content in the honey samples are in the range of 0.735 to 1.243 mg/g. The sample DK-Mi-03 had the highest amount of protein and the sample DK-De-02 had the least amount. Other than sample DK-Mi-03 ,samples DK-Bl-01, DK-Pa-04 and DK-Mu-07 had high protein content (more than 1 mg /g).The protein that pollen provides is very important for the brood production and development of young bees. Pollen is also the main source of protein to honey bees (Ellis *et al*., 2010).The antioxidant content in the samples varied from 200 to 323 mg/g . Honey sample DK-Pa-04 had the highest amount of antioxidant and sample DK-Pu-06 had the least amount. In a study conducted by Ferreira *et al* (2009), the antioxidant values ranged from 106.67 to 168.94 mg/ml or g .The results obtained by Bertoncelj *et al* (2007) ranged from 7.2 to 53.8 mg/ml.

A colorimetric assay using Folin Ciocalteu reagent is often referenced for the determination of phenolic compounds (Bertoncelj *et al*., 2007; Socha *et al*., 2009; Ferrieira *et al*., 2009; Beretta *et al*., 2005; Kaskoniene *et al*.,2009). The phenolic concentration in this study ranged from 817 to 1611 mg of gallic acid per kg. The honey sample with highest amount of phenolic content was DK-Pa-04 and sample with lowest content was DK-Mu-07. Evaluating honey, Ferreira et al (2009) reported phenol content ranging from 226.16 to 727.77 mg of gallic acid per kg of honey. Beretta et al. (2005) reported contents ranging from 52.2 to 789.6 mg per kg of honey. The sample DK-Pa-04 had the highest amount of flavanoid i.e. 737 mg/Kg of honey and sample DK-Bl-01 had the least amount which was 166 mg/Kg of honey.

REFERENCES

Babu, M.J., S.M. Ankolekar & K.P. Rajashekhar (2011). Castes of the weaver ant Oecophylla smaragdina (Fabricius) differ in the organization of sensilla on their antennae and mouthparts. Current Science 101(6): 755–764.

Barth, O.M. (1989). O polen no mel brasileiro, Grafica Luxor, (1-180).

Bayo, S.F., & Goka, K. (2014). Pesticide Residues and Bees - A Risk Assessment. Plos one. DOI: 10.1371/journal.pone.0094482. https://www.researchgate.net/publication/261520427.

Belden, J.B., Gilliom, R.J., Lydy, M.J. (2007). How well can we predict the toxicity of pesticide mixtures to aquatic life? Integrated Environmental Assessment and Management, 3, 364–372. DOI: 10.1002/ieam.5630030307.

Beretta, G., Granata, P., Ferrero, M., Orioli, M., & Facino, R.M. (2005). Standardization of antioxidant properties of honey by a combination of spectrophotometric/fluorimetric assays and chemometrics. Analytica Chimica Acta, 533, 185-191.

Bertoncelj, J., Dobersek, U., Jamnik, M., & Golob, T. (2007). Evaluation of phenolic content, antioxidant activity and colour of Slovenian honey. Food Chemistry, 105, 822-828.

Bonmatin JM, Marchand PA, Charvet R, Moineau I, Bengsch ER, et al. (2005) Quantification of imidacloprid uptake in maize crops. J Agric Food Chem 53: 5336–5341.

Calderone, N. W., & Page, R. E. (1992). Effects of interactions among genetically diverse nestmates on task specialization by foraging honey bees (Apis mellifera). Behavioral Ecology and Sociobiology, 30(4), 219-226.

Callahan, P.S. (1975). Insect antennae with special reference to the mechanism of scent detection and the evolution of the sensilla. International Journal of Insect Morphology and Embryology 4(5): 381–430. https://doi.org/10.1016/0020-7322(75)90038-0

Chang, C. C., Yang, M. H., Wen, H. M., & Chern, J. C. (2002). Estimation of total flavonoid content in propolis by two complementary colorimetric methods. Journal of Food and Drug Analysis, 10, 178-182.

Ellis, Amanda, Jamie D. Ellis, Michael K .O' Malley, and Catherine M. ZettelNalen. (2010), The benefits of pollen to honey bees. ENY152, UF/IFAS Extension.

Erban, T., & Hubert, J. (2010). Determination of pH in regions of the midguts of acaridid mites. Journal of Insect Science, 10(1), 42. https://doi.org/10.1673/031.010.4201

Ferreire, I.C.F.R., Aires, E., Barreira, J.C.M., & Estevinho, L. M. (2009). Antioxidant activity of Portuguese honey samples: Different contributions of the entire honey and phenolic extract. Food Chemistry, 114, 1438-1443.

Georgiadis, P., Pistorius, J., & Schwabe, K. (2012). Dust drift during sowing of maize - effects on honey bees. Environmental Science, Agricultural and Food Sciences. Julius-Kühn-Archiv. DOI: 10.5073/JKA.2012.437.039. Corpus ID: 82098315.

Gheldof, N., Engeseth, N. J. (2002). Antioxidant Capacity of Honeys from Various Floral Sources Based on the Determination of Oxygen Radical Absorbance Capacity and Inhibition of in Vitro Lipoprotein Oxidation in Human Serum Samples." Journal of Agricultural and Food Chemistry, 50(10), pp. 3050-3055. DOI: 10.1021/jf0114637.

Karnovsky, M. (1964). A Formaldehyde-Glutaraldehyde fixative of high osmolality for use in electron microscopy. Journal of Cell Biology 27: 1A–149A.

Kaskoniene, V., Maruska, A., & Kornysova, O. (2009). Quantitative and qualitative determination of phenolic compounds in honey. Chemine Technologija, 52(3), 74-80.

Kubik M, Nowacki J, Pidek A, Warakomska Z, Michalczuk L, et al. (1999) Pesticide residues in bee products collected from cherry trees protected during blooming period with contact and systemic fungicides. Apidologie 30: 521–532.

Kumar, R. & N.R. Kumar (2016). Scanning electron microscopic study on the mouthparts of Apis florea (Hymenoptera: Apidae). Apidologie 47(5): 717–727. https://doi.org/10.1007/s13592-015-0423-5

Lacerda, J.J.J., Santos, J.S., Santos, S.A., Rodrigues, G.B., & Santos, M.L.P. (2010). Influencia das caracteristicas fisico-quimicas e composicao elementar nas cores de meis produzidos por Apis mellifera no sudoeste da Bahia utilizando analise multivariada. Quimica Nova, 33(5), 1022-1026.

Lee, S. C., & Lee, S. J. (2014). Uptake of liquid from wet surfaces by the brush-tipped proboscis of a butterfly. Scientific Reports, 4, 6934.

Louveaux, J., A. Maurizio & G. Vorwhol (1978). Method of melissopalynology. Bee World, 59, 139–157.

Lowry O.H., Rosebrough N.J. , Farr A.L., Randall R.J.(1951). Protein measurement with the Folinphenol reagent, Journal of Biological chemistry, 193, 265-275.

Marinelli, E., Pulcini, P., Margio, C., De Pace, F., Allegrini, F., & Persona Oddo, L. (2004). Oxalic acid by Varrox® to Varroa control in Central Italy. Apiacta, 39, 39-43.

McCune, F., Samson-Robert, O., Rondeau, S., Chagnon, M., & Fournier, V. (2021). Supplying honey bees with waterers: a precautionary measure to reduce exposure to pesticides. Environmental Science and Pollution Research. DOI: 10.1007/s11356-020-12147-3.

Miller, H. (2021). The importance of bees to humans, the planet, and food supplies, Medical news today.

Orantes-Bermejo, F. J., Pajuelo, A. G., Megías, M. M. & Fernández-Píñar, C. T. (2010). Pesticide residues in beeswax and beebread samples collected from honey bee colonies (Apis mellifera L.) in Spain: Possible implications for bee losses. Journal of Apicultural Research, 48: 243-250. doi: 10.3896/IBRA.1.49.3.03

Ponnuchemy, R., V. Bonhomme, S. Prasad, L. Das, P. Patel, C. Gauchrel, A. Pragasam & K. Anupama (2014). Honey pollen: using melissopalynology to understand foraging preferences of bees in tropical south India. PLoS ONE, 9(7), e101618.

Smith, G.P.W., Thompson, H.M., Hardy, A.R., Bew, M.H., Findlay, E., & Stevenson, J.H. (1994). Incidents of poisoning of honeybees (Apis mellifera) by agricultural pesticides in Great Britain 1981–1991. Volume 13, Issue 8, Pages 567-581.

Socha, R., Juszczak, L., Pietrzyk, S., & Fortuna, T. (2009). Antioxidant activity and phenolic composition of herb honey. Food Chemistry, 113, 568-574.

Song, X.Y., Y.F. Yao & W.D. Yang (2012). Pollen analysis of natural honeys from the central region Shanxi, north China. PLoS ONE, 7(11), e49545.

Tapparo, A., Giorio, C., Marzaro, M., Marton, D., Soldà, L., & Girolami, V. (2011). Rapid analysis of neonicotinoid insecticides in guttation drops of corn seedlings obtained from coated seeds. Journal of Environmental Monitoring.

Turkmen, N., Sari, F., Poyrazoglu, E. S., & Velioglu, Y. S. (2006). Effect of prolonged heating on antioxidant activity and colour of honey. Food Chemistry, 95(4), 653-657. DOI: 10.1016/j.foodchem.2005.02.004.

Whitcomb, W. & H.F. Wilson (1929). Mechanics of digestion of pollen by the adult honey bee and the relation of undigested parts to dysentery of bees. Research Bulletin Agriculture Experiment. State University of Wisconsin Madison, 92, 1–27.

White, J.W., Maher J.(1980).Hidroxymethylfurfural content of honey as an indicator of its adulteration with invert sugars, Bee World, 61,29-37

White J.W. (1975). Physical characteristics of honey. In: Crane, E. (ed.), Honey, a Comprehensive Survey, Heinemann, London, Uk 207–239.

Whitehead, A.T. & J.R. Larsen (1976b). Ultrastructure of the contact chemoreceptors of Apis mellifera L. (Hymenoptera: Apidae). International Journal of Insect Morphology and Embryology 5(4): 301–315. https://doi.org/10.1016/0020-7322(76)90030-1

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